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(54) Title: *IN VIVO* USE OF RECOMBINAGENIC OLIGONUCLEOBASES TO CORRECT GENETIC LESIONS IN HEPATOCYTES

(57) Abstract

The present invention concerns compositions and methods for the introduction of specific genetic changes in endogenous genes of the cells of an animal. The genetic changes are effected by oligonucleotides or oligonucleotide derivatives and analogs, which are generally less than about 100 nucleotides in length. The invention provides for macromolecular carriers, optionally incorporating ligands for clathrin coated pit receptors. In one embodiment the ligand is a lactose or galactose and the genetic changes are made in hepatocytes. By means of the invention up to 40 % of the copiies of a target gene have been changed *in vitro*. Repair of mutant genes having a Crigler–Najjar like phenotype and Hemophilia B phenotype were observed.

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IN VIVO USE OF RECOMBINAGENIC OLIGONUCLEOBASES TO CORRECT GENETIC LESIONS IN HEPATOCYTES

This application claims benefit of the priority of U.S. patent application Serial No. 60/045,288, filed April 30, 1997, Serial No. 60/054,837, filed August 5, 1997, Serial No. 60/064,996, filed November 10, 1997, each of which are hereby incorporated by reference in their entirety.

1. FIELD OF THE INVENTION

The invention concerns methods and compositions for the use of recombinagenic oligonucleobases *in vivo* for the correction of disease causing genetic defects. The invention is particularly suited for the treatment of genetic defects of the type where the correction of the genetic defect in the hepatocytes of the subject will ameliorate the disease.

2. BACKGROUND TO THE INVENTION

2.1 THE USE OF CHIMERIC MUTATIONAL VECTORS TO EFFECT GENETIC CHANGES IN CULTURED CELLS

The inclusion of a publication or patent application in this section is not an admission that the publication or the invention, if any, of the application occurred prior to the present invention or resulted from the conception of a person other than the present inventors.

The published examples of recombinagenic oligonucleobases are termed Chimeric Mutational Vectors (CMV) or chimeraplasts because they contain both 2'-O-modified ribonucleotides and deoxyribonucleotides.

An oligonucleotide having complementary deoxyribonucleotides and ribonucleotides and containing a sequence homologous to a fragment of the bacteriophage M13mp19, was described in Kmiec, E.B., et al., November 1994, Mol. and Cell. Biol. **14**, 7163-7172. The oligonucleotide had a single contiguous segment of ribonucleotides. Kmiec et al. showed that the oligonucleotide was a substrate for the REC2 homologous pairing enzyme from *Ustilago maydis*.

Patent publication WO 95/15972, published June 15, 1995, and counterpart U.S. Patent No. 5,565,350 (the '350 patent) described duplex CMV for the introduction of genetic changes in eukaryotic cells. Examples in a *Ustilago maydis* gene and in the murine ras gene were reported. The latter example was designed to introduce a transforming mutation into the ras gene so that the successful mutation of the ras gene in NIH 3T3 cells would cause the growth of a colony of cells ("transformation"). The '350 patent reported that the maximum rate of transformation of NIH 3T3 was less than 0.1 %, i.e., about 100 transformants per 10^6 cells exposed to the ras duplex CMV. In the *Ustilago maydis* system the rate of transformants was about 600 per 10^6. A chimeric vector designed to introduce a mutation into a human bcl-2 gene was described in Kmiec, E.B., February 1996, Seminars in Oncology **23**, 188.

A duplex CMV designed to repair the mutation in codon 12 of K-ras was described in Kmiec, E.B., December 1995, Advanced Drug Delivery Reviews **17**, 333-40. The duplex CMV was tested in Capan 2, a cell line derived from a human pancreatic adenocarcinoma, using LIPOFECTIN™ to introduce the duplex CMV into the Capan 2 cells. Twenty four hours after the duplex CMV were introduced, the cells were harvested and genomic DNA was extracted; a fragment containing codon 12 of K-ras was amplified by PCR and the rate of conversion estimated by hybridization with allele specific probes. The rate of repair was reported to be approximately 18%.

A duplex CMV designed to repair a mutation in the gene encoding liver/bone/kidney type alkaline phosphatase was reported in Yoon, K., et al., March 1996, Proc. Natl. Acad. Sci. **93**, 2071. The alkaline phosphatase gene was transiently introduced into CHO cells by a plasmid. Six hours later the duplex CMV was introduced. The plasmid was recovered at 24 hours after introduction of the duplex CMV and analyzed. The results showed that approximately 30 to 38% of the alkaline phosphatase genes were repaired by the duplex CMV.

WO 97/41411 by E.B. Kmiec, A. Cole-Strauss and K. Yoon, and the publication Cole-Strauss, A., et al., Sept 1996, SCIENCE **273**, 1386 disclose duplex CMV that are used in the treatment of genetic diseases of hematopoietic cells, e.g., Sickle Cell Disease, Thalassemia and Gaucher Disease. WO 97/48714 and counterpart United States Patent No. 5,731,181 by E.B. Kmiec describes duplex CMV having non-natural

nucleotides for use in specific, site-directed mutagenesis. The duplex CMV described in the applications and certain of the publications of Kmiec and his colleagues contain a central segment of DNA:DNA homoduplex and flanking segments of RNA:DNA heteroduplex or 2'-OMe-RNA:DNA heteroduplex. Kren et al., 1997, Hepatology **25,** 1462-1468, reports the successful use of a CMV in non-replicating primary hepatocytes. Kren et al., March 1998, Nature Medicine **4,** 285 report the use of a CMV *in vivo* to introduce a genetic defect in the gene encoding blood coagulation factor IX in a rat.

The work of Kmiec and his colleagues concerned cells that are mitotically active, i.e., proliferating cells, at the time they are exposed to CMV. Kmiec and colleagues used a CMV/liposomal macromolecular carrier complex in which the CMV were mixed with a pre-formed liposome or lipid vesicle. In such a complex the CMV are believed to adhere to the surface of the liposome.

2.2 The Use of a Polyethylenimine Macromolecular Carrier For *In Vivo* and *In Vitro* Transfection

Branched chain polyethylenimine has been used as a carrier to introduce nucleic acids into eukaryotic cells both *in vivo* and *in vitro*. Boussif, O., et al., 1995, Proc. Natl. Acad. Sci. **92**, 7297; Abdallah, B. et al., 1996, Human Gene Therapy 7, 1947. Boletta, A., et al., 1997, **8,** 1243-1251. The *in vitro use of* galactosylated polyethylenimine to introduce DNA into cultured HepG2 hepatocarcinoma cell lines is reported by Zanta, et al., October 1, 1997, Bioconjugate Chemistry **8,** 839-844. The coupling of a protein ligand, transferrin, to polyethylenimine and its use to introduce a test gene into cultured cells by use of the transferrin receptor is described in Kircheis, R., et al., 1997, Gene Therapy **4,** 409-4-18. Branched chain polyethylenimines contain secondary and tertiary amino groups having a broad range of pK's and, consequently these polyethylenimines have a substantial buffering capacity at a pH where polylysine has little or no capacity, i.e., less than about 8. Tang, M.K., & Szoka, F.C., 1997, Gene Therapy **4,** 823-832.

The successful *in vivo* and *in vitro* use of linear polyethylenimine to transfect a gene is reported by Ferrari, S., et al., 1997, Gene Therapy **4,** 1100-1106.

2.3 THE USE OF A LIPOSOMAL CARRIER FOR *IN VIVO* TRANSFECTION

The use of liposomes or lipid vesicles to introduce DNA encoding a foreign protein into cells has been described. The most frequently used techniques adhere the DNA to the surface of a positively charged liposome, rather than encapsulating the DNA, although encapsulated DNA techniques were known. United States Patent Nos. 4,235,871 and 4,394,448 are relevant. The field is reviewed by Smith, J.G., et al., 1993, Biochim. Biophy. Acta **1154**, 327-340 and Staubinger, R.M., et al., 1987, Methods in Enzymology **185**, 512. The use of DOTAP, a cationic lipid in a liposome to transfect hepatic cells *in vivo* is described in Fabrega, A.J., et al., 1996, Transplantation **62**, 1866-1871. The use of cationic lipid-containing liposomes to transfect a variety of cells of adult mice is described in Zhu, N., et al., 1993, Science **261**, 209. The use of phosphatidylserine containing lipids to form DNA encapsulating liposomes for transfection is described in Fraley, R., et al., 1981, Biochemistry **20**, 6978-87.

2.4 THE USE OF THE ASIALOGLYCOPROTEIN RECEPTOR FOR HEPATOCELLULAR SPECIFIC TRANSFECTION

United States Patent Nos. 5,166,320 and 5,635,383 disclose the transfection of hepatocytes by forming a complex of a DNA, a polycationic macromolecular carrier and a ligand for the asialoglycoprotein receptor. In one embodiment, the macromolecular carrier was polylysine. The use of a lactosylcerebroside containing liposome to transfect a hepatocyte *in vivo* is described by Nandi, P.K., et al., 1986, J. Biol. Chem. **261**, 16722-16722. The use of asialofetuin-labeled liposomes to transfect liver cells with a reporter plasmid is described in Hara et al., 1995, Gene Therapy **2**, 764-788.

3. SUMMARY OF THE INVENTION

The present invention concerns compositions comprising a recombinagenic oligonucleobase and methods of their use to introduce specific genetic alterations in the DNA of the cells of a subject individual, a process termed "transmutation." A recombinagenic oligonucleobase is a nucleobase polymer that contains fewer than 60 bases of the sequence of the gene that is the target of the transmutation. The invention

can be used with any recombinagenic oligonucleobase now known or to be developed. In one embodiment the recombinagenic nucleobase is a Chimeric Mutational Vector (CMV) and the process of using CMV to effect transmutation is termed "Chimeraplasty." In contrast to previous such compositions and methods, the compositions and methods of the invention can be administered to the subject individual to effect the genetic alteration *in vivo*. The present invention depends in part on the unexpected discovery that CMV are effective to transmutate non-proliferating, *i.e.*, non-mitotic, cells. The invention further depends upon the unexpected discovery that CMV complexed with a macromolecular carrier to which is attached a ligand for a cell-surface receptor that is internalized through clathrin-coated pits into endosomes are suitable for chimeraplasty (a "clathrin-coated pit receptor").

In particular embodiments, the invention provides a composition comprising a CMV and a macromolecular carrier selected from the group consisting of: an aqueous-cored lipid vesicle of between 25 nm and 400 nm diameter, wherein the aqueous core contains the CMV; a lipid nanosphere of between 25 nm and 400 nm diameter, having a lipid core, wherein the lipid core contains a lipophilic salt of the CMV; and a polycationic salt of the CMV. Examples of polycations for such salts include polyethylenimine, polylysine and histone H1. In one embodiment the polycation is a branched chain polyethylenimine (PEI) salt having a mass average molecular weight greater than 500 daltons and less than 1.3 Md.

The carrier can be optionally substituted with a ligand for a cellular receptor that is internalized through a clathrin-coated pit into an endosome. In one embodiment the ligand is a lactosyl moiety and the receptor is the asialoglycoprotein receptor of the hepatocyte. Other receptors include the receptors for transferrin, insulin, glucagon, EGF and low density lipoproteins (LDL) and the ligand can be a fragment of the natural ligand that binds the receptor. Alternatively, the ligand can be any compound that binds specifically to the receptor, whether related to a naturally occurring ligand or otherwise. When administered to a subject individual the composition of the CMV/macromolecular carrier/ligand further comprises a pharmaceutically acceptable aqueous carrier, such as a buffered saline solution. When administered to a cell culture

the composition further comprises cell culture medium, which can be supplemented with serum.

A further embodiment of the invention comprises the administration of the above described complex to a human subject having a disease-causing genetic condition. The complex can be administered parenterally and in a preferred embodiment the complex is administered directly to the organ that is affected by the disease-causing genetic condition. In a yet further embodiment, the invention comprises the use of the CMV/macromolecular carrier/ligand complex for chimeraplasty in a subject individual and in cell culture.

4. DEFINITIONS

The invention is to be understood in accordance with the following definitions.

An oligonucleobase is a polymer of nucleobases, which polymer can hybridize by Watson-Crick base pairing to a DNA having the complementary sequence.

Nucleobases comprise a base, which is a purine, pyrimidine, or a derivative or analog thereof. Nucleobases include peptide nucleobases, the subunits of peptide nucleic acids, and morpholine nucleobases as well as nucleosides and nucleotides. Nucleosides are nucleobases that contain a pentosefuranosyl moiety, e.g., an optionally substituted riboside or 2'-deoxyriboside. Nucleosides can be linked by one of several linkage moieties, which may or may not contain a phosphorus. Nucleosides that are linked by unsubstituted phosphodiester linkages are termed nucleotides.

A oligonucleobase chain has a single 5' and 3' terminus, which are the ultimate nucleobases of the polymer. A particular oligonucleobase chain can contain nucleobases of all types. An oligonucleobase compound is a compound comprising one or more oligonucleobase chains that are complementary and hybridized by Watson-Crick base pairing. Nucleobases are either deoxyribo-type or ribo-type. Ribo-type nucleobases are pentosefuranosyl containing nucleobases wherein the 2' carbon is a methylene substituted with a hydroxyl, alkyloxy or halogen. Deoxyribo-type nucleobases are nucleobases other than ribo-type nucleobases and include all nucleobases that do not contain a pentosefuranosyl moiety.

An oligonucleobase strand generically includes both oligonucleobase chains and segments or regions of oligonucleobase chains. An oligonucleobase strand has a 3' end and a 5' end. When a oligonucleobase strand is coextensive with a chain, the 3' and 5' ends of the strand are also 3' and 5' termini of the chain.

A region is a portion of an oligonucleobase, the sequence of which is derived from some particular source, e.g., a CMV having a region of at least 15 nucleotides having the sequence of a fragment of the human ß-globin gene. A segment is a portion of a CMV having some characteristic structural feature. A given segment or a given region can contain both 2'-deoxynucleotides and ribonucleotides. However, a ribo-type segment or a 2'-deoxyribo-type segment contain only ribo-type and 2'-deoxyribo-type nucleobases, respectively.

5. DETAILED DESCRIPTION OF THE INVENTION

In sections 5.2 et seq. below, it should be understood that the teaching with regard to CMV is applicable to recombinagenic oligonucleobases as well.

5.1 THE STRUCTURE OF THE CHIMERIC MUTATIONAL VECTOR

The Chimeric Mutational Vectors (CMV) are comprised of polymers of purines and pyrimidines that hybridize, i.e., form Watson-Crick base pairs of purines and pyrimidines, to DNA having the appropriate sequence. Each CMV is divided into a first and a second strand of at least 15 bases each that are complementary to each other and can be, but need not be, covalently linked. The subunits of the oligonucleobase polymers are termed nucleobases. Nucleobases contain a base, which is either a purine or a pyrimidine or analog or derivative thereof. There are two types of nucleobases. Ribo-type nucleobases are ribonucleosides having a 2'-hydroxyl, substituted hydroxyl or 2'-halo-substituted ribose. All nucleobases other than ribo-type nucleobases are deoxyribo-type nucleobases.

The sequence of the first and second strands consists of at least two regions that are homologous to the target gene and one or more regions (the "mutator regions") that differ from the target gene and introduce the genetic change into the target gene. The mutator region is directly adjacent to homologous regions in both the 3' and 5'

directions. In a preferred embodiment of the invention, each mutator region is adjacent in both the 3' and 5' directions to homologous regions of at least three bases. In a preferred embodiment of the invention, each mutator region is flanked in both the 3' and 5' directions by ribo-type oligonucleobase segments of at least three bases, which segments need not be adjacent to the mutator region. The flanking ribo-type nucleobase segments need not be directly adjacent to the mutator region, i.e, a portion of the homologous region comprising deoxyribo-type nucleobases can intervene. The total length of all homologous regions is preferably at least 16 bases and is more preferably from about 20 nucleotides to about 60 nucleotides in length. If the CMV contains two homologous regions separated by a mutator region, the homologous regions can more preferably be each between 8 and 15 bases long and most preferably be 10 bases long.

At least two homologous regions of the first strand are comprised of at least three contiguous ribo-type nucleobases which are Watson-Crick paired to deoxyribo-type nucleobases of the second strand. In a preferred embodiment there are between 9 and 25 ribo-type nucleobases and more preferably 20 ribo-type nucleobases in the first strand, which are Watson-Crick paired to deoxyribo-type nucleobases of the second strand. In one embodiment there are no ribo-type nucleobases in the second strand. In one embodiment the mutator region of the first strand consists of deoxyribo-type nucleobases and is flanked by deoxyribo-type nucleobases. Alternatively, the mutator region can be comprised of ribo-type nucleobases of the first strand and deoxyribo-type nucleobases of the second strand.

Preferably the mutator region consists of 20 or fewer bases, more preferably 6 or fewer bases and most preferably 3 or fewer bases. The mutator region can be of a length different than the length of the sequence that separates the regions of the target gene homology with the homologous regions of the CMV so that an insertion or deletion of the target gene results. When the CMV is used to introduce a deletion in the target gene there is no base identifiable as within the mutator region. Rather, the mutation is effected by the juxtaposition of the two homologous regions that are separated in the target gene. For the purposes of the invention, the length of the mutator region of a CMV that introduces a deletion in the target gene is deemed to be

the length of the deletion. In one embodiment the mutator region is a deletion of from 6 to 1 bases or more preferably from 3 to 1 bases. Multiple separated mutations can be introduced by a single CMV, in which case there are multiple mutator regions in the same CMV. Alternatively multiple CMV can be used simultaneously to introduce multiple genetic changes in a single gene or, alternatively to introduce genetic changes in multiple genes of the same cell. Herein the mutator region is also termed the heterologous region.

In one embodiment the CMV is a single oligonucleobase chain of between 20 and 200 nucleobases and preferably between 40 and 100 nucleobases. In an alternative embodiment, the CMV comprises a first and a second oligonucleobase chain, each of between 20 and 100 bases; wherein the first chain comprises the first strand and the second chain comprises the second strand. The first and second chains can be linked covalently by other than nucleobases or, alternatively, can be associated only by Watson-Crick base pairings. Covalent linkage of the first and second strands can be made by oligo-alkanediols such as polyethyleneglycol, poly-1,3-propanediol or poly-1,4-butanediol.

In an alternative embodiment the invention can be practiced using CMV comprising deoxynucleotides or deoxynucleosides and 2'-O substituted ribonucleotides or ribonucleosides. Suitable substituents include the substituents taught by the Kmiec Application, C_{1-6} alkane. Alternative substituents include the substituents taught by U.S. Patent No. 5,334,711 (Sproat) and the substituents taught by patent publications EP 629 387 and EP 679657 (collectively, the Martin Applications), which are hereby incorporated by reference. As used herein a 2' fluoro, chloro or bromo derivative of a ribonucleotide or a ribonucleotide having a substituted 2'-O as described in the Martin Applications or Sproat is termed a "2'-Substituted Ribonucleotide." Particular preferred embodiments of 2'-Substituted Ribonucleotides are 2'-fluoro, 2'-methoxy, 2'-propyloxy,

2'-allyloxy, 2'-hydroxylethyloxy, 2'-methoxyethyloxy, 2'-fluoropropyloxy and 2'-trifluoropropyloxy substituted ribonucleotides. In a more preferred embodiments of 2'-Substituted Ribonucleotides are 2'-fluoro, 2'-methoxy, 2'-methoxyethyloxy, and 2'-allyloxy substituted nucleotides.

2'-Substituted Ribonucleosides are defined analogously. Particular preferred embodiments of 2'-Substituted Ribonucleosides are 2'-fluoro, 2'-methoxy, 2'-propyloxy, 2'-allyloxy, 2'-hydroxylethyloxy, 2'-methoxyethyloxy, 2'-fluoropropyloxy and 2'-trifluoropropyloxy substituted ribonucleotides. In a more preferred embodiments of 2'-Substituted Ribonucleosides are 2'-fluoro, 2'-methoxy, 2'-methoxyethyloxy, and 2'-allyloxy substituted nucleotides.

The term "nuclease resistant ribonucleoside" encompasses 2'-Substituted Ribonucleosides, including 2'-Substituted Ribonucleotides and also all 2'-hydroxyl ribonucleosides other than ribonucleotides. In a preferred embodiment , the CMV preferably includes at least three and more preferably six nuclease resistant ribonucleosides. In one preferred embodiment the CMV contains no nuclease sensitive ribonucleosides. In an alternative preferred embodiment, every other ribonucleoside is nuclease resistant. Certain 2'-blocking groups can be more readily synthesized for purines or pyrimidines. In one embodiment of the CMV only the ribonucleoside purines or only the ribonucleoside pyrimdines are nuclease resistant.

The CMV is still further characterized by containing at least three nuclease resistant ribo-type nucleobases. In a preferred embodiment all ribo-type nucleobases are nuclease resistant.

5.2 Formulations Suitable For *In Vivo* Use

The prior art formulations of CMV and a macromolecular carrier are of limited utility for *in vivo* use because of their low capacity for CMV and because the CMV is not protected from extracellular enzymes. The invention provides three alternative macromolecular carriers that overcome the limitations of the prior art. The carriers are polyethylenimine (PEI), aqueous-cored lipid vesicles, which are also termed unilamellar liposomes and lipid nanospheres.

Each of the carriers can be further provided with a ligand that is complementary to a cell-surface protein of the target cell. Such ligands are useful to increase both the amount and specificity of the uptake of CMV into the targeted cell. In one embodiment of the invention the target cell is a hepatocyte and the ligand is a galactose saccharide or lactose disaccharide that binds to the asialoglycoprotein receptor.

5.2.1 Polycationic Carriers

The invention can be practiced using any polycation that is non-toxic when administered to cells *in vitro* or to subjects *in vivo*. Suitable examples include polybasic amino acids such as polylysine, polyarginine, basic proteins such as histone H1, and synthetic polymers such as the branched-chain polyethylenimine:

$$(\text{-NHCH}_2\text{CH}_2\text{-})_X\,[\text{-N(CH}_2\text{CH}_2\text{NH}_2)\text{CH}_2\text{CH}_2\text{-}]_Y.$$

The invention can be practiced with any branched chain polyethylenimine (PEI) having an average molecular weight of greater than about 500 daltons, preferably greater than between about 10 Kd and more preferably about 25 Kd (mass average molecular weight determined by light scattering) The upper limit of suitability is determined by the toxicity and solubility of the PEI. Toxicity and insolubility of molecular weights greater than about 1.3 Md makes such PEI material less suitable. The use of high molecular weight PEI as a carrier to transfect a cell with DNA is described in Boussif, O. et al., 1995, Proc. Natl. Acad. Sci. **92**, 7297, which is hereby incorporated by reference. PEI solutions can be prepared according to the procedure of Boussif et al.

The CMV carrier complex is formed by mixing an aqueous solution of CMV and a neutral aqueous solution of PEI at a ratio of between 9 and 4 PEI nitrogens per CMV phosphate. In a preferred embodiment the ratio is 6. The complex can be formed, for example, by mixing a 10 mM solution of PEI, at pH 7.0 in 0.15 M NaCl with CMV to form a final CMV concentration of between 100 and 500 nM.

In addition a ligand for a clathrin-coated pit receptor can be attached to the polycation or to a fraction of the polycations. In one embodiment the ligand is a saccharide or disaccharide that binds to the asialoglycoprotein receptor, such as lactose, galactose, or N-acetylgalactosamine. Any technique can be used to attach the

ligands. The optimal ratio of ligand to polyethylene subunit can be determined by fluorescently labeling the CMV and injecting fluorescent CMV/molecular carrier/ligand complexes directly into the tissue of interest and determining the extent of fluorescent uptake according to the method of Kren et al., 1997, Hepatology **25,** 1462-1468.

Good results can be obtained using a 1:1 mixture of lactosylated PEI having a ratio of 0.4-0.8 lactosyl moieties per nitrogen and unmodified PEI. The mixture is used in a ratio of between 4 and 9 PEI nitrogens per CMV phosphate. A preferred ratio of oligonucleotide phosphate to nitrogen is 1:6. Good results can be obtained with PEIs having a mass average molecular weight of 25 Kd and 800 Kd which are commercially available from Aldrich Chemical Co., Catalog No. 40,872-7 and 18,197-8, respectively.

In an alternative embodiment the polycationic carrier can be a basic protein such as histone H1, which can be substituted with a ligand for a clathrin-coated pit receptor. A 1:1 (w/w) mixture of histone and CMV can be used to practice the intention.

5.2.2 Lipids that Are Useful in Carriers

The selection of lipids for incorporation into the lipid vesicle and lipid nanosphere carriers of the invention is not critical. Lipid nanospheres can be constructed using semi-purified lipid biological preparations, e.g., soybean oil (Sigma Chem. Co.) egg phosphatidyl choline (EPC) (Avanti Polar Lipids). Other lipids that are useful in the preparation of lipid nanospheres and/or lipid vesicles include neutral lipids, e.g., dioleoyl phosphatidylcholine (DOPC), and dioleoyl phosphatidyl ethanolamine (DOPE), anionic lipids, e.g., dioleoyl phosphatidyl serine (DOPS) and cationic lipids, e.g., dioleoyl trimethyl ammonium propane (DOTAP), dioctadecyldiamidoglycyl spermine (DOGS), dioleoyl trimethyl ammonium (DOTMA) and DOSPER (1,3-di-oleoyloxy-2-(6-carboxy-spermyl)-propyl-amide tetraacetate, commercially available from Boehringer-Mannheim). Additional examples of lipids that can be used in the invention can be found in Gao, X. and Huany, L., 1995, Gene Therapy 2, 710. Saccharide ligands can be added in the form of saccharide cerebrosides, e.g., lactosylcerebroside or galactocerebroside (Avanti Polar Lipids).

The particular choice of lipid is not critical. Hydrogenated EPC or lysolecithin can be used in place of EPC. DPPC (dipalmitoyl phosphatidylcholine), can be incorporated to improve the efficacy and/or stability of the delivery system.

5.2.3 The Construction of Lipid Nanosphere Carriers

Lipid nanospheres can be constructed by the following process. A methanol or chloroform methanol solution of phospholipids is added to a small test tube and the solvent removed by a nitrogen stream to leave a lipid film. A lipophilic salt of CMV is formed by mixing an aqueous saline solution of CMV with an ethanolic solution of a cationic lipid. Good results can be obtained when the cationic species are in about a 4 fold molar excess relative to the CMV anions (phosphates). The lipophilic CMV salt solution is added to the lipid film, vortexed gently followed by the addition of an amount of neutral lipid equal weight to the phospholipids. The concentration of CMV can be up to about 3% (w/w) of the total amount of lipid.

After addition of the neutral lipid, the emulsion is sonicated at 4°C for about 1 hour until the formation of a milky suspension with no obvious signs of separation. The suspension is extruded through polycarbonate filters until a final diameter of about 50 nm is achieved. When the target cell is a reticuloendothelial cell the preferred diameter of the lipid nanospheres is about 100-200 nm. The CMV-carrying lipid nanospheres can then be washed and placed into a pharmaceutically acceptable carrier or tissue culture medium. The capacity of lipid nanospheres is about 2.5 mg CMV/ 500 μl of a nanosphere suspension.

5.2.4 The Construction of Lipid Vesicles

A lipid film is formed by placing a chloroform methanol solution of lipid in a tube and removing the solvent by a nitrogen stream. An aqueous saline solution of CMV is added such that the amount of CMV is between 20% and 50% (w/w) of the amount of lipid, and the amount of aqueous solvent is about 80% (w/w) of the amount of lipid in the final mixture. After gentle vortexing the liposome-containing liquid is forced through successively finer polycarbonate filter membranes until a final diameter of about 50 nm is achieved. The passage through the successively finer polycarbonate

filter results in the conversion of polylamellar liposomes into unilamellar liposomes, i.e., vesicles. When the target cell is a reticuloendothelial cell the preferred diameter of the lipid nanospheres is about 100-200 nm. The CMV-carrying lipid nanospheres can then be washed and placed into a pharmaceutically acceptable carrier or tissue culture medium.

The CMV are entrapped in the aqueous core of the vesicles. About 50% of the added CMV is entrapped.

A variation of the basic procedure comprises the formation of an aqueous solution containing a PEI/CMV condensate at a ratio of about 4 PEI imines per CMV phosphate. The condensate can be particularly useful when the liposomes are positively charged, i.e., the lipid vesicle contains a concentration of cations of cationic lipids such as DOTAP, DOTMA or DOSPER, greater than the concentration of anions of anionic lipids such as DOPS. The capacity of lipid vesicles is about 150 μg CMV per 500 μl of a lipid vesicle suspension.

In a preferred embodiment the lipid vesicles contain a mixture of the anionic phospholipid, DOPS, and a neutral lipid such as DOPE or DOPC. Alternative, negatively charged phospholipids that can be used to make lipid vesicles include dioleoyl phosphatidic acid (DOPA) and dioleoyl phosphatidyl glycerol (DOPG). In a more preferred embodiment the neutral lipid is DOPC and the ratio of DOPS:DOPC is between 2:1 and 1:2 and is preferably about 1:1. The ratio of negatively charged to neutral lipid should be greater than 1:9 because the presence of less than 10% charged lipid results in instability of the lipid vesicles because of vesicle fusion.

A particular lipid vesicle formulation can be tested by using the formulation to transfect a target cell population with a plasmid of about 5.0 kb in length that expresses some readily detectable product in the transfected target cell. Lipid vesicles can be used to transfect a cell with the plasmid if the plasmid is condensed with PEI at an imine:phosphate ratio of about 9-4:1. The capacity of the lipid vesicle formulation to transfect a cell with a plasmid is indicative of the formulation's capacity to introduce a CMV into a cell and effect a transmutation.

Certain lipids, particularly the polycationic lipids, can be toxic to certain cell lines and primary cell cultures. The formulation of the lipid vesicles should be adjusted to avoid such toxic lipids.

Ligands for clathrin-coated pit receptors can be introduced into the lipid vescicles by a variety of means. Cerebrosides, such as lactocerebroside or galactocerebroside can be intorduced into the lipid mixture and are incorporated into the vesicle to produce a ligand for the asialoglycoprotein receptor.

In an alternative embodiment the lipid vesicle further comprise a integral membrane protein that inserts itself into the lipid bilayer of the vesicle. In a specific embodiment the protein is a fusigenic (F-protein) from the virus alternatively termed Sendai Virus or Hemagglutinating Virus of Japan (HVJ). The preparation and use of F-protein containing lipid vesicles to introduce DNA into liver, myocardial and endothelial cells have been reported. See, e.g., U.S. Patent No. 5,683,866, International Application PCT JP97/ 00612 (published as WO 97/31656). See also, Ramani, K., et al., 1996, FEBS Letters **404,** 164-168; Kaneda, Y., et al., 1989, J. Biol. Chem. **264,** 121126-12129; Kenada, Y., et al., 1989, Science **243,** 375; Dzau, V.J., et al., Proc. Natl. Acad. Sci. **93,** 11421-11425; Aoki, M., et al., 1997, J.Mol.Cardiol. **29,** 949-959.

5.3 Diseases and Disease-Specific CMV

The invention can be used to correct any disease-causing mutation, in which the mutation results in the change of one or more nucleotides or in the insertion or deletion of from one to about 30 nucleotides. In a preferred embodiment the deletion or insertion is of from one to about six nucleotides. The disease-causing mutation is corrected by administering a CMV containing the sequence of the wild type gene that is homologous to the locus of the mutation. The CMV is constructed so that there are regions of homology with the mutant DNA sequence flanking the heterologous region, i.e, the region of the CMV that contains the portion of the wild-type sequence that is absent from the mutant. When the mutation consists of an insertion, the heterologous region of the CMV is considered to be the point which is homologous to the site of the insertion. Accordingly, the length of the heterologous region of the CMV is deemed to

be the length of the insertion in the mutant sequence. Note that the sequence of the CMV is determined by the location of the mutation, however, the sequence of the mutation is not important. Rather, the sequence of a CMV is always the sequence of the wild type gene or a desired related sequence. In each of the sequences that follow the heterologous region is underlined.

A first embodiment of the invention is a CMV that can be used to correct the mutation that causes the von Willebrand's Disease. A CMV to correct this mutation contains the sequence 5'-CTC GGA GAG C CCC CTC GCA-3' (SEQ ID No. 1), the sequence of a mixed ribo-deoxyribo oligonucleobase having the same sequence of bases or a sequence that differs by the substitution of the thymine by uracil, or the sequence of the complement thereof. The tissue in which the von Willebrand's factor gene needs to be corrected is the vascular endothelium.

A further embodiment of the invention is a CMV that can be used to correct the mutation that causes Hemophilia B, which is an A→C substitution at nt 1234 of the human coagulation Factor IX gene. CMV to correct this mutation contains the sequence 5'-CAA GGA GAT AGT GGG GGA C-3' (SEQ ID No. 2), the sequence of a mixed ribo-deoxyribo oligonucleobase having the same sequence of bases or a sequence that differs by the substitution of the thymine by uracil, or the sequence of the complement thereof. The invention can be used to correct other mutations in the human coagulation Factor IX gene, the sequence of which is given in Kurachi, K., et al., 1982, Proc. Natl. Acad. Sci. U.S.A. **79**, 6461-6464, which is hereby incorporated by reference. The tissue in which the factor IX gene needs to be corrected is the hepatocellular liver.

A further embodiment of the invention is a CMV that can be used to correct the Z-mutation that causes α1-antitrypsin deficiency. The Z mutation is a G→A substitution located at nt 1145 of the human α1-antitrypsin gene. CMV to correct this mutation contains the sequence 5'-ACC ATC GAC GAG AAA GGG A-3' (SEQ ID No. 3), the sequence of a mixed ribo-deoxyribo oligonucleobase having the same sequence of bases or a sequence that differs by the substitution of the thymine by uracil, or the sequence of the complement thereof. The invention can be used to correct other mutations in the α1-antitrypsin gene, the sequence of which is given in Long, G.L., et

al., 1984, Biochemistry **23,** 4828-4837, which is hereby incorporated by reference. The tissue in which the α1-antitrypsin gene needs to be corrected is the hepatocellular liver.

A further embodiment of the invention is a CMV that can be used to correct a mutation in the low density lipoprotein receptor (LDLR) that causes familial hypercholesterolemia (FH). There is no single mutation that causes the majority of FH cases. Surveys of the more than 105 point mutations or insertions or deletions of 25 nt or fewer that cause FH can be found in Hobbs, H.H., et al., 1992, Hum. Mutat. **1**, 445-466 and Leren, T.P., et al., Hum. Genet. **95**, 671-676, which are hereby incorporated by reference in their entirety. The complete sequence of the human LDLR cDNA is published in Yamamoto, T., et al., 1984, CELL **39,** 27-38. The tissue in which the LDLR can be corrected to obtain amelioration of FH is the hepatocellular liver.

A further embodiment of the invention is a CMV that can be used to correct a mutation in the glucocerebrosidase gene that causes Gaucher Disease. The structure of CMV that can be used to correct a Gaucher Disease mutation can be found in commonly licensed U.S. application Serial No. 08/640,517. The tissue in which the glucocerebrosidase mutation can be corrected to obtain amelioration of Gaucher Disease is the reticuloendothelial (Kupffer Cell) liver.

A further embodiment of the invention is a CMV that can be used to correct a mutation in the glucose-6-phosphatase (G-6-P) gene that causes type 1 Glycogen Storage Disease (GSD). The complete sequence of the human G-6-P is given in Lei, K.-J., et al., SCIENCE **262,** 580, which is hereby incorporated by reference. The two most common mutations that cause type 1 GSD are C→T at nt 326, C→T at nt 1118, and an insertion of TA at nt 459, as described in Lei, K.-J., et al., J. Clin. Investigation **95,** 234-240, which is hereby incorporated by reference. CMV to correct the two most common mutations contain the sequence 5'-TTT GGA CAG CGT CCA TAC T-3' (SEQ ID No. 4), or 5'-TGC CTC GCC CAG GTC CTG G-3' (SEQ ID No. 5), the sequence of a mixed ribo-deoxyribo oligonucleobase having the same sequence of bases or a sequence that differs by the substitution of the thymine by uracil, or the sequence of the complement thereof.

A further embodiment of the invention is a CMV that can be used to correct a mutation in the Ornithine Transcarbamylase (OTC) gene an X-linked gene that catalyzes the condensation of ornithine and carbamyl phosphate to yield citruline and phosphate. The complete sequence of the human OTC cDNA is given in Horwich, A.L., et al., 1984, Science **224, 1068,** which is hereby incorporated by reference. The structure of OTC gene and a review of the structure of identified mutants is reviewed in Tuchman, M., 1992, Human Mutation **2,** 174.

A further embodiment of the invention is a CMV that can be used to correct a mutation in the human UDP-glucuronosyltransferase gene that causes Crigler-Najjar syndrome. The sequence of the human UDP-glucuronosyltransferase gene is given in Bosma, P.J., et al., 1992, Hepatology, 15, 941-7, which is hereby incorporated by reference. The tissue in which the UDP-glucuronosyltransferase gene can be corrected to obtain amelioration of Crigler-Najjar syndrome is the hepatocellular liver.

A further embodiment of the invention is a CMV that can be used to correct a mutation in a galactose-1-phosphate uridyltransferase gene that cause galactosemia. The sequence of the human galactose-1-phosphate uridyltransferase gene is described in Flack, J.E., et al., 1990 Mol. Biol. Med. **7,** 365, and the molecular biology and population genetics of galactosemia are described in Reichert, J.K.V., et al., 1991, Proc. Natl. Acad. Sci. **88,** 2633-37 and Reichert, J.K.V., et al., 1991, Am. J. Hum. Gen. **49,** 860, which are hereby incorporated by reference. The most common mutation that causes galactosemia is Q→R at amino acid 188. The CMV to correct this mutation contains the sequence 5'-CC CAC TGC CAG GTA TGG GC-3' (SEQ ID No. 6), the sequence of a mixed ribo-deoxyribo oligonucleobase having the same sequence of bases or a sequence that differs by the substitution of the thymine by uracil, or the sequence of the complement thereof.

A further embodiment of the invention is a CMV that can be used to correct a mutation in the phenylalanine hydroxylase (PAH, phenylalanine 4-monooxygenase, EC 1.14.16.1) that cause phenylketonuria (PKU) or hyperphenylalaninemia. The molecular and population genetics of phenylketonuria are described in Woo, SLC, 1989, Biochemistry **28**, 1-7, the sequence of human PAH is described in Kowk, S.C.M., et al., 1985, Biochemistry **28**, 556-561, which are hereby incorporated by reference. Further

examples of PKU-causing mutations can be found in Sworniczak, B., et al., 1992, Hum. Mutat. **1**, 138-146.

Those skilled in the art appreciate that the present invention can be used to make beneficial alterations in "normal" genes, where the identity of such beneficial alteration has been identified. For example, as many as 1 in 3,000 persons have serum cholesterol levels of 100 mg/dl or less because such individuals are heterozygous for a truncated lipoprotein apo B gene. Kane, J..P. and Havel, R.J., in THE METABOLIC BASIS OF INHERITED DISEASE, VOL. II (1995, McGraw Hill, New York) p. 1866. As used herein a "disease-causing mutation" includes examples such as the apo B gene, wherein the "normal" gene is associated with a disease, i.e., atherosclerosis, and a rare allele, in heterozygous form, is protective from that disease. Accordingly, in these circumstances, the statistically most prevalent gene should be regarded as the "disease-causing gene" and the gene having the beneficial alteration should be regarded as the "wild-type gene."

5.4 THE USE OF THE FORMULATIONS *IN VIVO*

The CMV of the invention can be parenterally administered directly to the target organ at a dose of between 50 and 250 μg/gm. When the target organ is the liver muscle or kidney, the CMV/macromolecular carrier complex can be injected directly into the organ. When the target organ is the liver, intravenous injection into the hepatic or portal veins of a liver, having temporarily obstructed circulation can be used. Alternatively the CMV/macromolecular complex can further comprise a hepatic targeting ligand, such as a lactosyl or galactosyl saccharide, which allows for administration of the CMV/macromolecular complex intravenously into the general circulation.

When the target organ is the lung or a tissue thereof, e.g., the bronchiolar epithelium CMV/macromolecular complex can be administered by aerosol. Small particle aerosol delivery of liposomal/DNA complexes is described in Schwarz L.A., et al., 1996, Human Gene Therapy 7, 731-741.

When the target organ is the vascular endothelium, as for example in von Willebrand's Disease, the CMV/macromolecular complex can be delivered directly into

the systemic circulation. Other organs can be targeted by use of liposomes that are provided with ligands that enable the liposome to be extravasated through the endothelial cells of the circulatory system.

For enzymatic defects, therapeutic effects can be obtained by correcting the genes of about 1% of the cells of the affected tissue. In a tissue in which the parenchymal cells have an extended life, such as the liver, treatments with CMV can be repeatedly performed to obtain an increased therapeutic effect.

6. EXAMPLES

6.1 CMV/MACROMOLECULAR CARRIER COMPLEXES

6.1.1 Lipid Nanospheres

Materials

Egg phosphatidylcholine (EPC), DOTAP and galactocerebroside (Gc) (Avanti Polar Lipids); soybean oil (Sigma Chemical Co.); dioctadecyldiamidoglycyl spermine (DOGS®) (Promega).

Methods

EPC, DOTAP and Gc were previously dissolved at defined concentrations in chloroform or anhydrous methanol and stored in small glass vials in desiccated containers at -20°C until use. EPC (40-45 mg), DOTAP (200 μg) and Gc (43 μg) solutions were aliquoted into a small 10 x 75 mm borosilicate tube and solvents removed under a stream of nitrogen. CMV were diluted in 0.15 M NaCl (~80-125 μg/250-300μl); DOGS (as a 10 mg/ml solution in ethanol) was diluted into 250-300 μl 0.15 M NaCl at 3-5 times the weight of added CMV. The two solutions were mildly vortexed to mix contents and then CMV solution was added slowly to the DOGS solution. The contents were mixed by gentle tapping and inverting the tube a few times. The DOGS-complex solution was added to the dried lipids followed by soybean oil (40-45 mg), the mixture was vortexed on high for a few seconds and bath sonicated in a FS-15 (Fisher Scientific) bath sonicator for ~1 hr in a 4°C temperature controlled room. Occasionally, the tube was removed from the bath and vortexed. When a uniform looking, milky suspension was

formed (with no obvious separation of oil droplets), it was extruded through a series of polycarbonate membranes down to a pore size of 50 nm. Preparations were stored at 4°C until use and vortexed before use.

6.1.2 Negatively Charged Targeted Lipid Vesicles

Materials

Dioleoyl phosphatidylcholine (DOPC), dioleoyl phosphatidylserine (DOPS), galactocerebroside (Gc) or lactosylcerebroside, (Avanti Polar Lipids).

Methods

DOPS, DOPC and Gc at a molar ratio of 1:1:0.16 (500 μg total lipid) were dissolved in chloroform:methanol (1:1 v/v) and then dried under a stream of nitrogen to obtain a uniform lipid film. The CMV were diluted in 500 μl of 0.15 M NaCl (approximately 100-250 μg/500μl). The solution was added to the lipid film at room temperature. Lipids were dispersed entirely by alternate mild vortexing and warming (in a water bath at 37-42°C). After a uniform milky suspension was formed, it was extruded through a series of polycarbonate membranes (pore sizes 0.8, 0.4, 0.2, 0.1 and 0.05μm) using a Liposofast® mini-extruder. Extrusion was done 5 to 7 times through each pore size. After preparation, lipid vesicles were stored at 4°C until use. Under these conditions the lipid vesicles were stable for at least one month. The final product can be lyophilized.

6.1.3 Neutral Targeted Lipid Vesicles

Materials

Dioleoyl phosphatidylcholine (DOPC), dioleoyl phosphatidylethanolamine (DOPE), galactocerebroside (Gc) or lactosylcerebroside, (Avanti Polar Lipids).

Methods

DOPC, DOPE and Gc (1:1:0.16 molar ratio) or DOPC:Gc (1:0.08) were dissolved in chloroform:methanol (1:1 v/v) and then dried under a stream of nitrogen to obtain a uniform lipid film. The oligonucleotides (or chimeric molecules) were diluted in 500 μl

of 0.15 M NaCl (approximately 100-250 μg/500 μl). The solution was added to the lipid film at room temperature. Lipids were dispersed entirely by alternate mild vortexing and warming (in a water bath at 37-42°C). After a uniform milky suspension was formed, it was extruded through a series of polycarbonate membranes (pore sizes 0.8, 0.4, 0.2, 0.1 and 0.05 μm) using a Liposofast® mini-extruder. Extrusion was done 5 to 7 times through each pore size. After preparation, lipid vesicles were stored at 4°C until use. The size of the lipid vesicles of the preparation was stable for about 5 days.

6.1.4 Positively Charged Targeted Lipid Vesicles

Materials

Dioleoyl phosphatidylcholine (DOPC), dioleoyl trimethylammonium propane (DOTAP), galactocerebroside (Gc) or lactosylcerebroside, (Avanti Polar Lipids). Polyethylenimine (PEI) (M.W. 800 Kd), Fluka Chemicals.

Methods

DOPC, DOTAP and Gc (6:1:0.56 molar ratio) (500 μg total lipid) were dissolved in chloroform:methanol (1:1 v/v) and then dried under a stream of nitrogen to obtain a uniform lipid film. PEI was diluted to a concentration of 45 mg/100 ml using water. pH of the solution was adjusted to ~7.6 using HCl. This PEI stock solution was prepared fresh each time and was equivalent to approximately 50 nmol amine/μl. CMV were diluted into 0.15 M NaCl at a concentration of ~125 μg in 250 μl. PEI was further diluted into 250 μl 0.15 M NaCl so that approximately 4 moles of PEI amine were present per mole of oligonucleotide/chimeric phosphate. PEI solution was added drop-wise to the CMV solution (both at room temperature) and vortexed for 5-10 minutes. The PEI-complex solution was then added to the lipid film and the lipids dispersed as described above. After a uniform milky suspension was formed, it was extruded through a series of polycarbonate membranes (pore sizes 0.8, 0.4, 0.2, 0.1 and 0.05 μm) using a Liposofast® mini-extruder. Extrusion was done 5 to 7 times through each pore size. After preparation lipid vesicles were stored at 4°C until use. Under these conditions the lipid vesicles were stable for at least one month. For longer and improved stability the final product can be lyophilized.

6.1.5 Lactosylated-PEI/PEI Complexes

PEI (25 kDa) was purchased from Aldrich Chemical (Milwaukee, WI). PEI (800 kDa) was purchased from Fluka chemicals (Ronkonkoma, NY, USA) . Lactosylation of the PEI was carried out by modification of a previously described method for the conjugation of oligosaccharides to proteins. Briefly, 3 to 5 ml of PEI (0.1 to 1.2 $M_{monomer}$) in ammonium acetate (0.2 M) or Tris buffer (0.2 M) (pH 7.6) solution was incubated with 7 to 8 mg of sodium cyanoborohydride (Sigma Chemical Co., St. Louis, MO) and approximately 30 mg of lactose monohydrate (Sigma Chemical Co., St. Louis, MO). Reaction was carried out in polypropylene tubes, tightly capped in a 37°C shaking water bath. After 10 days the reaction mixture was dialyzed against distilled water (500 ml) for 48 h with 1 to 2 changes of water. The purified complex was sterile filtered through 0.2 μm filter and stored at 4°C. The amount of sugar (as galactose) associated with PEI was determined by the phenol-sulphuric acid method.

The number of moles of free amine (primary + secondary) in the lactosylated PEI was determined as follows: a standard curve was set up using a 0.02M stock solution of PEI; several aliquots of the stock were diluted to 1ml using deionized water in glass tubes, then 50 μl of Ninhydrin reagent (Sigma Chemical Co., St. Louis, Mo) was added to each tube and vortexed vigorously for 10 sec. Color development was allowed to proceed at room temperature for 10 to 12 min. and then O.D. was read (within 4 minutes) at 485 nm on a Beckman DU-64 spectrophotometer. 20 to 50 μl aliquots of the L-PEI samples were treated as above and the number of moles of free amine was determined from the standard curve. Lactosylated-PEI (L-PEI) complexes were prepared as follows: an equivalent of 3 mmol of amine as L-PEI and 3 mmol of amine as PEI, per mmol of RNA/DNA phosphate, were mixed together and diluted in 0.15M NaCl as required; the mixture was added dropwise to a solution of the chimeric and vortexed for 5 min.

To verify complete association of the chimeric oligonucleotides with PEI or L-PEI, gel analysis (4% LMP agarose) of the uncomplexed and complexed chimerics was performed. To determine the degree of protection against nuclease degradation provided by complexation of the chimerics, samples were treated with RNAse and

DNAse. After a chloroform phenol extraction, the complexes were dissociated using heparin (50 units/μg nucleic acid) and the products analyzed on a 4% LMP agarose gel.

6.2 Demonstration of PEI/CMV Mediated Alteration of Rat and Human Factor IX

Materials. Fetal bovine serum was obtained from Atlanta Biologicals, Inc. (Atlanta, GA). The terminal transferase, fluorescein-12-dUTP, Expand™ high fidelity PCR system, dNTPs and high pure PCR template preparation kit were obtained from Boehringer Mannheim Corp. (Indianapolis, IN). Reflection™ NEF-496 autoradiography film and Reflection™ NEF-491 intensifying screens were from DuPont NEN® Research Products (Boston, MA). Polyethylenimine (PEI) 800 kDa was obtained from Fluka Chemical Corp. (Ronkonkoma, NY). The [γ-^{32}P]ATP was obtained from ICN Biochemicals, Inc. (Costa Mesa, CA). pCR™2.1 was obtained from Invitrogen (San Diego, CA). OPTIMEM™, Dulbecco's modified Eagle's medium, William's E medium and oligonucleotides 365-A and 365-C were from Life Technologies, Inc. (Gaithersburg, MD). Spin filters of 30,000 mol wt cutoff were purchased from Millipore Corp. (Bedford, MA). DiI and SlowFade™ antifade mounting medium were obtained from Molecular Probes, Inc. (Eugene, OR). T4 polynucleotide kinase was purchased from New England Biolabs, Inc. (Beverly, MA). MSI MagnaGraph membrane was purchased from Micron Separations, Inc. (Westboro, MA). The primers used for PCR amplification were obtained from Oligos Etc., Inc. (Wilsonville, OR). Tetramethylammonium chloride was purchased from Sigma Chemical Company (St. Louis, MO). All other chemicals were molecular biology or reagent grade and purchased from Aldrich Chemical Company (Milwaukee, WI), Curtin Matheson Scientific, Inc. (Eden Prairie, MN), and Fisher Scientific (Itasca, IL).

Oligonucleotide synthesis. Chimeric RNA/DNA oligonucleotides HIXF, RIXF and RIXR were synthesized. The CMV were prepared with DNA and 2'-O-methyl RNA phosphoramidite nucleoside monomers on an ABI 394 synthesizer. The DNA phosphoramidite exocyclic amine groups were protected with benzoyl (adenosine and cytidine) and isobutyryl (guanosine). The protective groups on the 2'-O-methyl RNA phosphoramidites were phenoxyacetyl for adenosine, isobutyryl for cytidine, and

dimethylformamide for guanosine. The base protecting groups were removed following synthesis by heating in ethanol/concentrated ammonium hydroxide for 20 h at 55°C. The crude oligonucleotides were electrophoresed on 15% polyacrylamide gels containing 7 M urea, and the DNA visualized using UV shadowing. The chimeric molecules were eluted from the gel slices, concentrated by precipitation and desalted using G-25 spin columns. Greater than 95% of the purified oligonucleotides were full length.

The sequence of the wild type and "mutant" rat Factor IX are

(SEQ ID No. 7)

```
                                    365
 wt  AAA GAT TCA TGT GAA GGA GAT AGT GGG GGA CCC CAT GTT
     Lys Asp Ser Cys Glu Gly Asp Ser Gly Gly Pro His Val
```

(SEQ ID No. 8)

(SEQ ID No. 9)

```
mt  AAA GAT TCA TGT GAA GGA GAT CGT GGG GGA CCC CAT GTT
                                  Arg
```

The structure of the RIXR, RIXF and HIXR CMV is as follows:

Chimeric Oligonucleotides

RIXR (SEQ ID No. 10)

```
 TGCGCG-ccccaggggg TGCTA gaggaaguguT
T                                    T
T                                    T
 TCGCGC GGGGTCCCCC ACGAT CTCCTTCACAT
       3' 5'
```

$RIXR_C$ (SEQ ID No. 11)

```
 TGCGCG-acacuuccuc TAGCA cccccuggggT
T                                    T
T                                    T
 TCGCGC TGTGAAGGAG ATCGT GGGGGACCCCT
       3' 5'
```

RIXF (SEQ ID No. 12)

TGCGCG-acacuuccuc**TAGCA**ccccccuggggT

T T

T T

TCGCGC TGTGAAGGAG**ATCGT**GGGGGACCCCT

3' 5'

HIXF (SEQ ID No. 13)

TGCGCG-acaguuccuc**TAGCA**ccccccuggggT

T T

T T

TCGCGC TGTCAAGGAG**ATCGT**GGGGGACCCCT

3' 5'

Uppercase letters are deoxyribonucleotides, lower case letters are 2'OMe-ribonucleotides. The nucleotide of the heterologous region is underlined.

Cell Culture, transfections and hepatocyte isolation. HuH-7 cells were maintained in Dulbecco's modified Eagle's medium containing 10% (vol/vol) heat inactivated fetal bovine serum in a humidified CO_2 atmosphere at 37°C. Twenty four hours prior to transfection 1 x 10^5 cells were plated per 35 mm culture dish. At the time of transfection, the cells were rinsed twice with OPTIMEM™ media and transfections were performed in 1 ml of the same media. Eighteen hours after transfection, 2 ml of Dulbecco's modified Eagle's medium containing 20% (vol/vol) heat inactivated fetal bovine serum was added to each 35 mm dish and the cells maintained for an additional 30 h prior to harvesting for DNA isolation. A PEI (800 kDa) 10 mM stock solution, pH 7.0, was prepared. Briefly, the chimeric oligonucleotides were transfected with 10 mM PEI at 9 equivalents of PEI nitrogen per chimeric phosphate in 100 μl of 0.15 M NaCl at final concentrations of either 150 nM (4 μg), 300 nM (8 μg) and 450 nM (12 μg). After 18 h, an additional 2 ml of medium was added and reduced the chimeric concentrations to 50 nM, 100 nM, and 150 nM, respectively, for the remaining 30 h of culture. HuH-7 vehicle control transfections utilized the same amount of PEI as was used in the HuIXF transfections, but substituted an equal volume of 10 mM Tris-HCl pH 7.6 for the oligonucleotides.

Primary rat hepatocytes were isolated from 250 g male Sprague-Dawley rats (Harlan Sprague-Dawley, Inc., Indianapolis, IN) by a two step collagenase perfusion as previously described (Fan et al., Oncogene 12:1909-1919, 1996, which is hereby incorporated by reference) and plated on Primaria™ plates at a density of 4 x 10^5 cells per 35 mm dish. The cultures were maintained in William's E medium supplemented with 10% heat inactivated FBS, 26 mM sodium bicarbonate, 23 mM HEPES, 0.01 U/ml insulin, 2 mM L-glutamine, 10 nM dexamethasone, 5.5 mM glucose, 100 U/ml penicillin and 100 U/ml streptomycin. Twenty four hours after plating, the hepatocytes were washed twice with the same medium and 1 ml of fresh medium added and the cells transfected using PEI/chimeric oligonucleotide complexes at the identical concentrations as for the HuH-7 cells. After 18 h, an additional 2 ml of the medium was added and the cells harvested 6 or 30 h later.

Direct injection of chimeric oligonucleotides into liver. Male Sprague-Dawley rats (~175 g) were maintained on a standard 12 h light-dark cycle and fed *ad libitum* standard laboratory chow. The rats were anesthetized, a midline incision made the liver exposed. A clamp was placed on the hepatic and portal veins as they enter the caudate lobe, and 75 μg of the 1:9 chimeric/PEI complex was injected in a final volume of 250 - 300 μl directly into the caudate lobe. The lobe remained ligated for 15 min and then blood flow was restored by removing the clamp. After suturing the incision the animals were allowed to recover from the anesthesia and given food and water *ad libitum*. Vehicle controls were done substituting an equal volume of Tris-HCl pH 7.6 for the chimeric oligonucleotides. Twenty-four and 48 h post-injection the animals were sacrificed, the caudate lobe removed and the tissue around the injection site dissected for DNA isolation. DNA was isolated and the terminal exon of the rat factor IX gene was amplified by PCR.

Nuclear uptake of the chimeric molecules. Chimeric duplexes were 3' end-labeled using terminal transferase and fluorescein-12-dUTP according to the manufacturer's recommendation, and were then mixed with unlabeled oligonucleotides at a 2:3 ratio. Transfections were performed as described above and after 24 h the cells were fixed in phosphate buffered saline, pH 7.4, containing 4% paraformaldehyde (wt/vol) for 10 min at room temperature. Following fixation, the cells were

counterstained using a 5 μM solution of DiI in 0.32 M sucrose for 10 min according to the manufacturer's recommendation. After washing with 0.32 M sucrose and then phosphate buffered saline, pH 7.4, the cells were coversliped using SlowFade™ antifade mounting medium in phosphate buffered saline and examined using a MRC1000 confocal microscope (BioRad, Inc., Hercules, CA). The caudate lobes of liver *in situ* were injected with fluorescently-labeled chimerics as described above and harvested 24 h post-injection. The lobes were bisected longitudinally, embedded using OCT and frozen. Cryosections were cut ~10 μm thick, fixed for 10 min at room temperature using phosphate buffered saline, pH 7.4, containing 4% paraformaldehyde (wt/vol). Following fixation, the cells were counterstained using a 5 μM solution of DiI in 0.32 M sucrose for 10 min according to the manufacturer's recommendation. After washing with 0.32 M sucrose and then phosphate buffered saline, pH 7.4, the sections were coversliped using SlowFade™ antifade mounting medium and examined using a MRC1000 confocal microscope (BioRad, Inc.). The collection series for the fixed cells and sectioned tissue were made at 1 μm steps to establish the presence of the chimeric in the nucleus.

DNA isolation and cloning. The cells were harvested by scrapping 24 and 48 h after transfection. Genomic DNA larger than 100-150 base pairs was isolated using the high pure PCR template preparation kit according to the manufacturer's recommendation. PCR amplification of a 317-nt fragment of the eighth exon in the human liver IX gene was performed with 500 ng of the isolated DNA. The primers used were 5′-CATTGCTGACAAGGAATACACGAAC-3′ (SEQ ID No. 14) and 5′-ATTTGCCTTTCATTGCACACTCTTC-3′ (SEQ ID No. 15) corresponding to nucleotides 1008-1032 and 1300-1324, respectively, of the human factor IX cDNA. Primers were annealed at 58°C for 20 sec, elongation was for 45 sec at 72°C and denaturation proceeded for 45 sec at 94°C. The sample was amplified for 30 cycles using Expand Hi-fidelity™ polymerase. PCR amplification of a 374-nt fragment of the rat factor IX gene was performed with 500 ng of the isolated DNA from either the primary hepatocytes or liver caudate lobe. The primers used were 5′-ATTGCCTTGCTGGAACTGGATAAC-3′ (SEQ ID No. 16) and 5′-TTGCCTTTCATTGCACATTCTTCAC-3′ (SEQ ID No. 17) corresponding to

nucleotides 433-457 and 782-806, respectively, of the rat factor IX cDNA. Primers were annealed at 59°C for 20 sec, elongation was for 45 sec at 72°C and denaturation proceeded for 45 sec at 94°C. The sample was amplified for 30 cycles using Expand Hi-fidelity™ polymerase. The PCR amplification products from both the human and rat factor IX genes were subcloned into the TA cloning vector pCR™2.1 according to the manufacturer's recommendations, and the ligated material used to transform frozen competent *Escherichia coli*.

Colony hybridization and sequencing. Eighteen to 20 h after plating, the colonies were lifted onto MSI MagnaGraph nylon filters, replicated and processed for hybridization according to the manufacturer's recommendation. The filters were hybridized for 24 h with 17 mer oligonucleotide probes 365-A (5'-AAGGAGATAGTGGGGGA-3') (SEQ ID No. 18) or 365-C (5'AAGGAGATCGTGGGGGA-3') (SEQ ID No. 19), where the underlined nucleotide is the target of the mutagenesis. The probes were ^{32}P-end-labeled using [γ-^{32}]ATP (>7,000 Ci/mmol) and T4 polynucleotide kinase according to the manufacturer's recommendations. Hybridizations were preformed at 37°C in 2X sodium chloride sodium citrate containing 1% SDS, 5X Denhardt's and 200 μg/ml denatured sonicated fish sperm DNA. After hybridization, the filters were rinsed in 1X sodium chloride phosphate EDTA, 0.5% SDS and then washed at 54°C for 1 h in 50 mM Tris-HCl, pH 8.0 containing 3 M tetramethylammonium chloride, 2 mM EDTA, pH 8.0, 0.1% SDS. Autoradiography was performed with NEN® Reflection film at -70°C using an intensifying screen. Plasmid DNA was prepared from colonies identified as hybridizing with 365-A or 365-C using Qiagen minprep kit (Chatsworth, CA) and subjected to automatic sequencing using the mp13 reverse primer on an ABI 370A sequencer (Perkin-Elmer, Corp., Foster City, CA).

Results *In Vivo*

Chimeric oligonucleotides were fluorescein-labeled and used to determine whether direct injection into the caudate lobe of the liver was feasible. The results indicated that the hepatocytes adjacent to the injection site within the caudate lobe showed uptake of the fluorescently-labeled chimeric molecules similar to that observed in

isolated primary hepatocytes and HuH-7 cells. Although some punctate material was present in the cytoplasm, the labeled material was detected primarily in the nucleus. In fact, only nuclear labeling was observed in hepatocytes farthest from the injection site. The unlabeled PEI/RIXF chimeric complexes and vehicle controls were injected directly into the caudate lobe using the same protocol and the animals sacrificed 24 and 48 h post-injection. Liver DNA was isolated as described in Methods, subjected to PCR amplification of a 374 nt sequence spanning the targeted nt exchange site. Following subcloning and transformation of *Escherichia coli* with the PCR amplified material, duplicate filter lifts of the transformed colonies were performed. The filters were hybridized with ^{32}-labeled 17-mer oligonucleotides specific for either 365-A (wild-type) or 365-C (factor IX mutation) and processed post-hybridization as described in Methods. Rats which received direct hepatic injection of the RIXF chimeric molecules exhibited a A→C conversion frequency of ~1% at both 24 and 48 h. In contrast, the vehicle controls showed no hybridization with the 365-C probe. Colonies that hybridized with the 365-C probe from the RIXF treated animals were cultured, the plasmid DNA isolated and subjected to sequencing to confirm the A→C conversion. The ends of the amplified 374-nt fragment correspond exactly with the primers and the only nucleotide change observed was an A→C at the targeted exchange site.

6.3 Demonstration of Lactosylated-PEI/CMV Mediated Alteration of Rat Factor IX

6.3.1 Results

CMV complexed to a mixture of lactosylated-PEI and PEI was prepared using the RIXR oligonucleotide as described in Section 6.1.5 above. A CMV directed to the complementary strand of the same region of the factor IX was also constructed ($RIXR_C$).

Conversion of the targeted nucleotide at Ser^{365} by the chimeric oligonucleotides

The nuclear localization of the fluorescently-labeled chimeric molecules indicated efficient transfection in the isolated rat hepatocytes. The cultured hepatocytes were then transfected with the unlabeled chimeric molecules factor $RIXR_C$ and RIXR at comparable concentrations using 800 kDa PEI as the carrier. Additionally, vehicle

control transfections were performed simultaneously. Forty eight hours after transfection, the cells were harvested and the DNA isolated and processed for hybridization as described in Section 6.1.5. The A→C targeted nucleotide conversion at Ser^{365} was determined by hybridization of duplicate colony lifts of the PCR-amplified and cloned 374-nt stretch of exon 8 of the factor IX gene (Sarkar, B., Koeberl, D. D. & Somer, S. S., "Direct Sequencing of the activation peptide and the catalytic domain of the factor IX gene in six species," *Genomics*, 6, 133-143, 1990.) The 17 mer oligonucleotide probes used to distinguish between the wild-type 365-A (5'-AAGGAGATAGTGGGGGA-3') (SEQ ID No. 20) or converted 365-C (5'-AAGGAGATCGTGGGGGA-3') (SEQ ID No. 21) corresponded to nucleotides 710 through 726 of the cDNA sequence.

The overall frequency of conversion of the targeted nucleotide was calculated by dividing the number of clones hybridizing with the 365-C oligonucleotide by the total number of clones hybridizing with both oligonucleotide probes. The results are summarized in Table 1 for $RIXR_C$. A→C conversion at Ser^{365} was observed only in primary hepatocytes transfected with the RIXR or $RIXR_C$. Similar conversion frequencies were observed in hepatocytes transfected with RIXR or $RIXR_C$. Neither vehicle transfected cells nor those transfected with other chimeric oligonucleotides yielded any clones hybridizing with the 365-C oligonucleotide probe (unpublished observations). Additionally, no hybridization of the 365-C oligonucleotide probe was observed to clones derived from DNA isolated from untreated hepatocytes and PCR-amplified in the presence of 0.5 to 1.5 μg of the oligonucleotides. The A→C conversion rate in the isolated hepatocytes was also dose dependent using lactosylated PEI derivatives as described in Section 6.1.5 and was as high as 19%. RT-PCR and hybridization analysis of RNA isolated from cultured cells transfected in parallel with lactosylated PEIs demonstrated A→C conversion frequencies ranging from 11.9 to 22.3%.

Site-directed nucleotide exchange by chimeric oligonucleotides in intact liver

The fluorescein-labeled oligonucleotides were also used to determine cellular uptake of the chimeric molecules after direct injection into the caudate lobe of the liver. The

results indicated that hepatocytes adjacent to the injection site in the caudate lobe showed uptake of the fluorescently-labeled chimerics similar to that observed in the isolated rat hepatocytes. Although some punctate material was present in the cytoplasm of the hepatocytes, the labeled material was primarily present in the nucleus. In fact, only nuclear labeling was observed in those areas farthest from the injection site. The unlabeled RIXR chimeric oligonucleotides and vehicle controls were then administered *in vivo* by tail vein injection of the 25 kDa PEI and liver tissue harvested 5 days post-injection. Liver DNA was isolated and subjected to PCR amplification of a 374-nt sequence spanning the targeted nucleotide exchange site, using the same primers as those used with the primary hepatocytes. Following subcloning and transformation of *E. coli* with the PCR-amplified material, duplicate filter lifts of the transformed colonies were done. The filters were hybridized with the same ^{32}P-labeled 17-mer oligonucleotides specific for either 365-A (wild-type) or 365-C (mutant) and processed post-hybridization. Rats treated with 100 μg of the RIXR chimeric oligonucleotides exhibited an A→C conversion frequency ranging from 13.9% to 18.9%, while those that received a total of 350 μg in two injections showed 40% conversion. In contrast, the vehicle controls showed no hybridization with the 365-C probe. RT-PCR hybridization of isolated RNA indicated A→C conversion frequencies of 26.4% to 28.4% in the high dose livers. The APTT for vehicle-treated rats ranged from 89.7% to 181.9% of control values (131.84% ± 32.89%), while the APTT for the oligonucleotide-treated animals ranged from 48.9% to 61.7% (53.8% ± 4.8%).

The APTT times for a 1/10 dilution of rat test plasma in Hepes buffer (50 mM Hepes/100 mM NaCl/0.02% NaN_3, pH 7.4) were determined for both normal (n = 9) and the double injected animals (n = 3). The factor IX activity of duplicate samples was determined from a log-log standard curve that was constructed from the APTT results for dilution (1:10 to 1:80) of pooled plasma from 12 normal male rats, 6-8 weeks old. The APTT results for the normal rats ranged from 89.7% to 181.9% of the control values (mean = 131.84% ± 32.89%), while the APTT results for the double injected animals ranged from 49.0% to 61.7% (mean 53.8% ± 5.8%). The APTT clotting time in seconds for the normal rats ranged from 60.9 seconds to 81.6 seconds (mean = 71.3

± 7.3 seconds) while the APTT times ranged from 92.3 seconds to 98.6 seconds (mean = 96.3 ± 2.9 seconds) for the double-infected rats.

Sequence analysis of the mutated factor IX gene in isolated hepatocytes and intact liver

Direct sequencing of the wild-type and mutated genes was performed to confirm the results from the filter hybridizations in both the *in vitro* and *in vivo* studies. At least 10 independent clones hybridizing to either 365-A or 365-C from the intact liver or isolated hepatocytes were analyzed. The results of the sequencing indicated that colonies hybridizing to 365-A (Fig. 6, top panel) exhibited the wild-type IX sequence, i.e. an A at Ser^{365} of the reported cDNA sequence. In contrast, those colonies derived from the factor $RIXR_C$ transfected primary hepatocytes hybridizing to the 365-C oligonucleotide probe converted to a C at Ser^{365}. The same A→C conversion at Ser^{365} was observed in the clones derived from the transfected rat liver that hybridized with the 17 mer 365-C oligonucleotide probe. The entire 374-nt PCR amplified region of the factor IX gene was sequenced for all the clones and no alteration other than the indicated changes at Ser^{365} was detected. Finally, the start and end points of the 374-nt PCR amplified genomic DNA derived from both the primary hepatocytes and the intact liver corresponded exactly to those of the primers used for the amplification process, indicating that the cloned and sequenced DNA was derived from genomic DNA rather than nondegraded chimeric oligonucleotides.

Table 1 Percent A→C conversion at Ser^{365} of rat factor IX genomic DNA by colony lift hybridizations

PEI Deliver System		365-C clones	Total clones	A→C (%)
PEI 800 kDa[1]	Concentration			
In vitro	150 nM	24	572	4.2
	300	31	367	8.5
	450	63	502	12.5
Lac-PEI 800 kDa				
In vitro	90	18	337	5.3
	180	34	300	11.3
	270	47	253	18.6
Lac-PEI 25 kDa				
In vitro	90	28	527	5.3
	180	53	417	12.7
	270	60	305	19.7
Lac-PEI 25 kDa[2]	Dose			
In vivo x1	100 μg	24	166	14.5
		71	386	18.4
		50	360	13.9
Lac-PEI 25 kDa				
In vivo x2	350 μg	237	601	39.4
		228	563	40.5
		271	678	40.0

[1]The data shown for the primary hepatocyte transfections represents a mean of two experiments.

[2]The *in vivo* chimeric/PEI complexes were administered in a volume of 300 μl of 5% dextrose by tail vein injection. The results of three animals at each dose are shown individually.

6.3.2 Materials and Methods

In vivo delivery of the chimeric oligonucleotides. Male Sprague-Dawley rats (Harlan Sprague-Dawley, Inc.) (~50 g) were maintained on a standard 12 h light-dark cycle and

fed *ad libitum* standard laboratory chow. Vehicle controls and lactosylated 25 kDa PEI at a ratio of 6 equivalents of PEI nitrogen per chimeric phosphate in 300 μl of 5% dextrose (Abdallah, B. et al., "A powerful nonviral vector for *in vivo* gene transfer into the adult mammalian brain: polyethylenimine:, *Human Gene Therapy*, 7, 1947-1954, 1996.). The aliquots were administered by tail vein injection either as a single dose of 100 μg or divided dose of 150 μg and 200 μg on consecutive days. Five days post-injection, liver tissue was removed for DNA and RNA isolation. DNA was isolated as previously described (Kren, B. T., Trembley, J. H. & Steer, C. J., "Alterations in mRNA stability during rat liver regeneration," *Am. J. Physiol.*, 270, G763-G777, 1996) for PCR amplification of exon 8 of the rat factor IX gene. RNA was isolated for RT-PCR amplification of the same region as the genomic DNA using RNAexol and RNAmate (Intermountian Scientific Corp., Kaysville, UT) according to the manufacturer's protocol.

Factor IX activity assay. Blood samples from vehicle (n = 9) and oligonucleotide-treated (n = 3) rats were collected 20 days after the second tail vein injection in 0.1 vol. of 0.105 M sodium citrate/citric acid. After centrifugation at 2,500 x g and then 15,000 x g the resulting plasma was stored at -70°C. The factor IX activity was determined from activated partial thromboplastin time (APTT) assays. Briefly, 50 μl of APTT reagent (DADE, Miami, FL), 50 μl of human factor IX-deficient plasma (George King Biomedical, Overland, KS), and 50 μl of 1/10 dilution of rat test plasma in Hepes buffer (50 mM Hepes/100 mM NaCl/0.02% NaN_3, pH 7.4) were incubated at 37°C for 3 min in an ST4 coagulometer (American Bioproducts, Parsippany, NJ). Clotting was initiated by addition of 50 μl of 33 mM $CaCl_2$ in Hepes buffer. Factor IX activity of duplicate samples was determined from a log-log standard curve constructed from the APTT results for dilution (1:10 to 1:80) of pooled plasma from normal male rats (n = 12).

DNA/RNA isolation and cloning. The cells were harvested by scrapping 48 h after transfection. Genomic DNA larger than 100-150 base pairs was isolated using the high pure PCR template preparation kit (Boehringer Mannheim, Corp., Indianapolis, IN).

RNA was isolated using RNAzol™ B (Tel-Test, Inc., Friendswood, TX), according to the manufacturer's protocol. PCR amplification of a 374-nt fragment of the rat factor IX gene was performed with 300 ng of the isolated DNA from either the primary hepatocytes or liver tissue. The primers were designed as 5'-ATTGCCTTGCTGGAACTGGATAAAC-3' (SEQ ID No. 22) and 5'TTGCCTTTCATTGCACATTCTTCAC-3' (SEQ ID No. 23) (Oligos Etc., Wilsonville, OR) corresponding to nucleotides 433-457 and 782-806, respectively, of the rat factor IX cDNA. Primers were annealed at 59°C for 20 sec, elongation was for 45 sec at 72°C and denaturation proceeded for 45 sec at 94°C. The sample was amplified for 30 cycles using Expand Hi-fidelity™ polymerase (Boehringer Mannheim, Corp.). The PCR amplification products from both the hepatocytes and intact liver factor IX genes were subcloned into the TA cloning vector pCR™2.1 (Invitrogen, San Diego, CA), and the ligated material used to transform frozen competent *E. coli*. To rule out PCR artifacts 300 ng of control DNA was incubated with 0.5, 1.0 and 1.5 μg of the oligonucleotide prior to the PCR-amplification reaction. Additionally, 1.0 μg of the chimeric alone was used as the "template" for the PCR amplification.

RT-PCR amplification was done utilizing the Titian™ one tube RT-PCR system (Boehringer Mannheim, Corp.) According to the manufacturer's protocol using the same primers as those used for the DNA PCR amplification. To rule out DNA contamination, the RNA samples were treated with RQ1 DNase free RNase (Promega Corp., Madison, WI) and RT-PCR negative controls of RNased RNA samples were performed in parallel with the RT-PCR reaction. Each of the PCR reactions were ligated into the same TA cloning vector and transformed into frozen competent *E. coli*.

Colony hybridization and sequencing. Eighteen to 20 h after plating, the colonies were lifted onto MSI MagnaGraph nylon filters, replicated and processed for hybridization according to the manufacturer's recommendation. The filters were hybridized for 24 h with 17 mer oligonucleotide probes 365-A (5'AAGGAGATAGTGGGGGA-3') (SEQ ID No. 24) OR 365-C (5'-AAGGAGATCGTGGGGGA-3') (SEQ ID No. 25) (Life technologies, Inc., Gaithersburg, MD), where the underlined nucleotide is the target for

mutagenesis. The probes were ^{32}P-end-labeled using (γ-^{32}P) ATP (>7,000 Ci/mmol) and T4 polynucleotide kinase (New England Biolabs, Inc., Beverly MA). Hybridizations were performed at 37°C in 2X sodium chloride sodium citrate containing 1% SDS, 5X Denhardt's and 200 μg/ml denatured sonicated fish sperm DNA. After hybridization, the filters were rinsed in 1X sodium chloride sodium phosphate EDTA, 0.5% SDS and then washed at 54°C for 1 h in 50 mM Tris-HCl, pH 8.0 containing 3 M tetramethylammonium chloride, 2 mM EDTA, pH 8.0, 0.1% SDS (Melchior, W. B. & Von Hippel, P. H. "Alteration of the relative stability of dA.dT and dG.dC base pairs in DNA," Proc. Natl. Acad. Sci. USA, **70,** 298-302, 1973.). Autoradiography was performed with NEN®Reflection film at -70°C using an intensifying screen. Plasmid DNA was prepared from colonies identified as hybridizing with 365-A or 365-C using Qiagen miniprep kit (Chatsworth, CA) and subjected to automatic sequencing using the mp13 forward and reverse primers as well as a gene specific primer, 5'GTTGACCGAGCCACATGCCTTAG-3' (SEQ ID No. 26) corresponding to nucleotides 616 to 638 of the rat factor IX cDNA using an ABI 370A sequencer (Perkin-Elmer, Corp., Foster City, CA).

6.4 Correction of a Hemophilia B Mutation in Chapel Hill Dogs

The Chapel Hill strain of dogs, which has a (G→A)^{1477} mutation that results in hemophilia in the animals, was used to obtain primary cultured hepatocytes. A CMV designed to correct the mutation is given below.

DIX1 (SEQ ID No. 28)

T T GCGCG au uca aag aaT TGA CCC TAA Taa ucg acc cc T T
T T CGCGC TA AGT TTC TTA ACT GGG ATT ATT AGC TGG GG T T
3' 5'

The hepatocytes were treated with 360 nM DIX1 complexed in either 25 kDa Lac-PEI or galactocerebroside-containing aqueous-cored, negatively charged lipid vesicles (Gc-NLV). The results are given in Table II below.

Frequency of conversion of A to G at nucleotide 1477 of the Factor IX Gene

(Primary Hepatocytes from the Chapel Hill Strain of Hemophilia B Dogs)

Vehicle	Number of Times Transfected	Concentration	G Clones/Total Clones	Frequency (%)
Gc-NLV	Once	360 nM	30/195	15.44
			30/218	13.76
	Twice		30/118	25.4
Lac-PEI 25 kDa	Once*	360 nM	20/141	14.2
			48/348	13.3
	Twice		21/107	19.6

*RT-PCR on parallel transfected cultures gave an A to G conversion frequency of 11.1%

6.5 Correction of a Crigler-Najjar-like Mutation in the Gunn Rat

Mutant rats with hyperbilirubinemia, termed Gunn rats, have a single nucleotide deletion in the gene encoding bilirubin-uridinediphosphoglucuronate glucuronosyltransferase (*UGT1A1*). Roy Chowdhury, J., et al., 1991, J. Biol. Chem. **266,** 18294. Human patients with Crigler-Najjar syndrome type I also have mutations of the *UGT1A1* gene, resulting in life-long hyperbilirubinemia and consequent brain damage. Bosma, P.J., et al., 1992, FASEB J. **6,** 2859; Jansen, P.L.M., et al., Progress In Liver Diseases, **XIII,** Boyer, J.L., & Ockner, R.K., editors (W.B. Saunders, Phil. 1995), pp 125-150. The structure of CN3, a CMV designed to correct the Gunn rat mutation is given below.

CN3 (mut→WT) (SEQ ID No. 27)

```
 T GCGCG gg gac uua caG GAC CTT TAC uga ctt cua T
T                                                 T
T                                                 T
 T CGCGC CC CTG AAT GTC CTG GAA ATG ACT GCC GAT T
       3' 5'
```

Gunn rat primary cultured hepatocytes were treated with 150 nM CN3 according to the above protocol except that the carrier was either the negatively charged glycosylated lipid vesicles of section 6.2.2 or a lactosylated-PEI carrier at a ratio of oligonucleotide phosphate to imine of 1:4 . The results were 8.5% conversion with the negatively charged liposome and 3.6% conversion with lactosylated-PEI carrier.

Gunn rats were injected with 1 mg/Kg of CN3 complexed with either 25 kDa Lac-PEI or complexed with negatively charged Gc lipid vesicles as described above. The rate of gene conversion was determined by cloning and hybridization according to the procedure described for factor IX. The results shown below indicate that between about 15% and 25% of the copies of the *UGT1A1* gene were converted.

Frequency of Insertion of G at nucleotide 1239 of the UGT-1 Gene (In Gunn Rats)

Vehicle	Dosage	G Clones/Total Clones	Frequency (%)
Gc-NLV	1 mg	112/815	15.4
		208/761	27.3
		185/974	18.9
		39/273	14.6[1]
		78/403	19.3[2]
25 kDa PEI. (Lactosylated)	1 mg	188/838	22.4
		254/1150	22.1
		245/997	24.6

[1]Initial conversion frequency determined.

[2]Conversion frequency determined 7 days after 70% partial hepatectomy.

A Gunn rat was injected on five successive days with 1mg/Kg of CN3 complexed with 25 kDa Lac-PEI as above. Twenty five days after the final injection the serum bilirubin had declined from 6.2 mg/dl to 3.5 mg/dl and remained at that level for a further 25 days.

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: Steer, Clifford J.
Kren, Betsy T.
Bandyopadhyay, Paramita T.

(ii) TITLE OF THE INVENTION: In Vivo Use of Recombinagenic Oligonucleobases to Correct Genetic Lesions in Hepatocytes

(iii) NUMBER OF SEQUENCES: 28

(iv) CORRESPONDENCE ADDRESS:
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(B) STREET: 300 Pheasant Run
(C) CITY: Newtown
(D) STATE: PA
(E) COUNTRY: USA
(F) ZIP: 18940

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ for Windows Version 2.0

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER:

(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Hansburg, Daniel

(B) REGISTRATION NUMBER: 36156

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(B) TELEFAX: 215-504-4545

(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTCGGAGAGC CCCCTCGCA 19

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CAAGGAGATA GTGGGGGAC 19

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ACCATCGACG AGAAAGGGA 19

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TTTGGACAGC GTCCATACT 19

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TGCCTCGCCC AGGTCCTGG 19

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCCACTGCCA GGTATGGGC 19

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AAAGATTCAT GTGAAGGAGA TAGTGGGGGA CCCCATGTT 39

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Lys Asp Ser Cys Glu Gly Asp Ser Gly Gly Pro His Val
1 5 10

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AAAGATTCAT GTGAAGGAGA TCGTGGGGGA CCCCATGTT 39

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 68 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GGGGTCCCCC ACGATCTCCT TCACATTTTU GUGAAGGAGA TCGTGGGGGA CCCCGCGCGT 60
TTTCGCGC 68

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 68 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TGTGAAGGAG ATCGTGGGGG ACCCCTTTTG GGGUCCCCCA CGATCUCCUU CACAGCGCGT 60
TTTCGCGC 68

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 68 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TGTGAAGGAG ATCGTGGGGG ACCCCTTTTG GGGUCCCCCA CGATCUCCUU CACAGCGCGT 60
TTTCGCGC 68

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 68 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TGTCAAGGAG ATCGTGGGGG ACCCCTTTTG GGGUCCCCCA CGATCUCCUU GACAGCGCGT 60
TTTCGCGC 68

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CATTGCTGAC AAGGAATACA CGAAC 25

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

ATTTGCCTTT CATTGCACAC TCTTC 25

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

ATTGCCTTGC TGGAACTGGA TAAC 24

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TTGCCTTTCA TTGCACATTC TTCAC 25

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

AAGGAGATAG TGGGGGA 17

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

AAGGAGATCG TGGGGGA 17

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

AAGGAGATAG TGGGGGA 17

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

AAGGAGATCG TGGGGGA 17

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

ATTGCCTTGC TGGAACTGGA TAAAC 25

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

TTGCCTTTCA TTGCACATTC TTCAC 25

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

AAGGAGATAG TGGGGGA 17

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

AAGGAGATCG TGGGGGA 17

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GTTGACCGAG CCACATGCCT TAG 23

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 76 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CCCTGAATGT CCTGGAAATG ACTGCCGATT TTTAUCTTCA GUCATTTCCA GGACAUUCAG 60
GGGCGCGTTT TCGCGC 76

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 80 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

TAAGTTTCTT AACTGGGATT ATTAGCTGGG GTTTTCCCCA GCUAATAATC CCAGTTAAGA 60

AACUUAGCGC GTTTTCGCGC

CLAIMS:

1. A composition comprising:
   a) a recombinagenic oligonucleobase;
   b) an aqueous carrier; and
   c) a macromolecular carrier selected from the group consisting of
      (i) an aqueous-cored lipid vesicle, wherein the aqueous core contains the oligonucleobase,
      (ii) a lipid nanosphere, which comprises a lipophilic salt of the oligonucleobase, and
      (iii) a polycation having an average molecular weight of between 500 daltons and 1.3 Md wherein the polycation forms a salt with the oligonucleobase.

2. The composition of claim 1, which further comprises a ligand for a clathrin-coated pit receptor, which receptor is linked to the macromolecular carrier.

3. The composition of claim 2, in which the macromolecular carrier is a negatively charged, aqueous-cored lipid vesicle.

4. The composition of claim 3, in which the aqueous-cored lipid vesicle comprises dioleoylphosphatidylcholine and dioleoylphosphatidylserine.

5. The composition of claim 4, in which the aqueous-cored lipid vesicle further comprises a cerebroside.

6. The composition of claim 2, wherein the macromolecular carrier is a branched-chain polyethylenimine.

7. The composition of claim 2, wherein the macromolecular carrier is an aqueous-cored lipid vesicle that comprises a fusigenic F-protein.

8. The composition of claim 2 in which the oligonucleobase comprises:

(i) a first and a second homologous region that are together at least 16 and not more than 60 nucleobases in length, which regions are homologous with a target gene of a mammal; and

(ii) a heterologous region that is disposed between the first and second homologous region and is at least 1 and not more than 20 nucleobases in length, which is heterologous with the target gene and which contains the alteration.

9. The composition of claim 8, in which the recombinagenic oligonucleobase comprises at least 15 deoxynucleotides that are Watson-Crick base paired with 2'-Substituted Ribonucleotides.

10. The composition of claim 9, in which the 2'-Substituted Ribonucleotides are independently selected from the group consisting of 2'-methoxy-ribonucleotides, 2'-allyloxy-ribonucleotides, 2'-methoxyethoxy-ribonucleotides and 2'-fluoro-ribonucleotides.

11. The composition of claim 8, in which the ligand is a ligand for a receptor selected from the group consisting of the receptors for transferrin, nicotinic acid, carnitine, insulin and insulin like growth factor-1.

12. The composition of claim 8, in which the clathrin-coated pit receptor is an asialoglycoprotein receptor.

13. The composition of claim 12, in which the ligand comprises a moiety selected from the group consisting of lactose, galactose, and N-acetylgalactosamine, and in which the sequence of the oligonucleobase comprises the sequence of a contiguous 16 nucleotide fragment of a human gene that encodes a product selected from the group consisting of α1-antitrypsin, coagulation factor IX, uridinediphosphoglucuronate glucuronosyltransferase, glucocerebrosidase,

glucose-6-phosphatase, low density lipoprotein receptor, ornithine transcarbamylase and phenylalanine hydroxylase or the complement thereof.

14. A method of altering a target gene in a tissue of a subject mammal comprising administering to the subject mammal a method comprising an aqueous, pharmaceutically acceptable carrier and a recombinagenic oligonucleobase, which olignucleobase comprises:

a) a recombinagenic oligonucleobase

b) an aqueous carrier; and

c) a macromolecular carrier selected from the group consisting of

(i) an aqueous-cored lipid vesicle, wherein the aqueous core contains the oligonucleobase,

(ii) a lipid nanosphere, which comprises a lipophilic salt of the oligonucleobase, and

(iii) a polycation having an average molecular weight of between 500 daltons and 1.3 Md wherein the polycation forms a salt with the oligonucleobase,

wherein a ligand for a clathrin-coated pit receptor is covalently linked to the macromolecular carrier.

15. The method of claim 14, wherein the tissue is the liver.

16. The method of claim 14, wherein the macromolecular carrier is a negatively charged, aqueous-cored lipid vesicle.

17. The method of claim 16, wherein the aqueous-cored lipid vesicle comprises dioleoylphosphatidyl-choline and dioleoylphosphatidylserine.

18. The method of claim 17 , wherein the aqueous-cored lipid vesicle further comprises a cerebroside.

19. The method of claim 14, wherein the macromolecular carrier is a branched-chain polyethylenimine.

20. The method of claim 14, wherein the macromolecular carrier is a linear polyethylenimine or a linear polycationic polypeptide.

21. The method of claim 14, wherein the oligonucleobase comprises:

a) a first and a second homologous region that are together at least 16 and not more than 60 nucleobases in length, which regions are homologous with a target gene of a mammal; and

b) a heterologous region that is disposed between the first and second homologous region and is at least 1 and not more than 20 nucleobases in length, which is heterologous with the target gene and which contains the alteration.

22. The method of claim 21, in which the recombinagenic oligonucleobase comprises at least 15 deoxynucleotides that are Watson-Crick base paired with 2'-Substituted Ribonucleotides.

23. The method of claim 22, wherein the 2'-Substituted Ribonucleotides are independently selected from the group consisting of 2'-methoxy-ribonucleotides, 2'-allyloxy-ribonucleotides, 2'-methoxyethoxy-ribonucleotides and 2'-fluoro-ribonucleotides.

24. The method of claim 22, wherein the clathrin-coated pit receptor is an asialoglycoprotein receptor and the macromolecular carrier is polyethylenimine or a negatively charged aqueous cored lipid vesicle.

25. A method of introducing a beneficial alteration in a disease-causing sequence of a target gene in a cell of a human subject which comprises administering to the subject an amount of a composition comprising an aqueous, pharmaceutically

acceptable carrier and an oligonucleobase having both ribo-type and deoxyribo-type nucleobases which oligonucleobase comprises:

a) a recombinagenic oligonucleobase

b) an aqueous carrier; and

c) a macromolecular carrier selected from the group consisting of

(i) an aqueous-cored lipid vesicle, wherein the aqueous core contains the oligonucleobase,

(ii) a lipid nanosphere, which comprises a lipophilic salt of the oligonucleobase, and

(iii) a polycation having an average molecular weight of between 500 daltons and 1.3 Md wherein the polycation forms a salt with the oligonucleobase,

wherein a ligand for a clathrin-coated pit receptor is covalently linked to the macromolecular carrier and wherein the amount is effective to ameliorate a disease caused by the sequence.

26. The method of claim 25, wherein the cell is a hepatocyte.

27. The method of claim 25, wherein the macromolecular carrier is a negatively charged, aqueous-cored lipid vesicle.

28. The method of claim 27, wherein the aqueous-cored lipid vesicle comprises dioleoylphosphatidylcholine and dioleoylphosphatidylserine.

29. The method of claim 28, wherein the aqueous-cored lipid vesicle further comprises a cerebroside.

30. The method of claim 25, wherein the macromolecular carrier is a branched-chain polyethylenimine.

31. The method of claim 25, wherein the macromolecular carrier is a linear polyethylenimine or a linear polycationic polypeptide.

32. The method of claim 25, wherein the oligonucleobase comprises:

a) a first and a second homologous region that are together at least 16 and not more than 60 nucleobases in length, which regions are homologous with a target gene of a mammal; and

b) a heterologous region that is disposed between the first and second homologous region and is at least 1 and not more than 20 nucleobases in length, which is heterologous with the target gene and which contains the alteration.

33. The method of claim 32, wherein the recombinagenic oligonucleobase comprises at least 15 deoxynucleotides that are Watson-Crick base paired with 2'-Substituted Ribonucleotides.

34. The method of claim 33, wherein the 2'-Substituted Ribonucleotides are independently selected from the group consisting of 2'-methoxy-ribonucleotides, 2'-allyloxy-ribonucleotides, 2'-methoxyethoxy-ribonucleotides and 2'-fluoro-ribonucleotides.

35. The method of claim 33, wherein the clathrin-coated pit receptor is an asialoglycoprotein receptor and the macromolecular carrier is polyethylenimine or a negatively charged, aqueous-cored lipid vesicle.

36. The method of claim 35, wherein the ligand comprises a moiety selected from the group consisting of lactose, galactose, and N-acetylgalactosamine, and wherein the sequence of the oligonucleobase comprises the sequence of a contiguous 16 nucleotide fragment of a human gene that encodes a product selected from the group consisting of α1-antitrypsin, coagulation factor IX, uridinediphosphoglucuronate glucuronosyltransferase, glucocerebrosidase,

glucose-6-phosphatase, low density lipoprotein receptor, ornithine transcarbamylase and phenylalanine hydroxylase or the complement thereof.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/08834

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C12Q 1/68; A61K 48/00; C07H 21/04
US CL :514/44; 435/6, 69.1, 172.3, 375; 536/23.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 435/6, 69.1, 172.3, 375; 536/23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG: Medline, Derwent Biotechnology Abstracts, CAS, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 94/12650 A2 (TRANSKARYOTIC THERAPIES, INC.) 09 June 1994, see entire document.	1-36
Y	WO 94/04032 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 03 March 1994, see entire document.	1-36
Y	CAPECCHI. Altering the genome by homologous recombination. Science. 16 June 1989, Vol. 244, pages 1288-1292, see entire document.	1-36
Y	US 5,264,618 A (FELGNER et al.) 23 November 1993, see entire document.	1-36

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"B"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
16 AUGUST 1998	24 SEP 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer John LeGuyader Telephone No. (703) 308-0196

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